Genetic Analysis of Fungicide-Resistant *Sclerotinia homoeocarpa* Isolates from Tennessee and Northern Mississippi

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ABSTRACT

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Sclerotinia homoeocarpa is the causal agent of dollar spot disease that reduces the uniformity and aesthetic value of golf putting greens. Fungicide-resistant isolates of *S. homoeocarpa* were collected from putting greens at 10 locations across Tennessee and northern Mississippi. Genetic diversity among the 60 isolates was investigated using vegetative compatibility, conserved gene sequences, and amplified fragment length polymorphism (AFLP). Six tester strains were paired with Tennessee and northern Mississippi isolates on potato dextrose agar. Some of the 60 isolates were delineated into vegetative compatibility groups, but fungicide resistance could not be associated with a particular vegetative compatibility group. Genetic similarities of isolates at the vegetative compatibility level could be attributed to founder effects. Sequencing the regions of CAD, EF1-α, β-tubulin, and internal transcribed spacers revealed 100% homology among isolates. Capillary gel electrophoresis and analysis of AFLP fragments indicated 86 to 100% similarity between the isolates. Vegetative compatibility and molecular data indicate that the populations of the pathogen are clonal. Isolates did not cluster according to fungicide resistance during unweighted pair group with arithmetic means analysis, but did appear to cluster according to vegetative compatibility group and location. Although associations could not be made between molecular markers and fungicide resistance, links between vegetative compatibility and AFLP markers may provide a foundation from which other studies could be performed.

Additional keywords: turfgrass disease

Sclerotinia homoeocarpa F.T. Benn., the causal agent of dollar spot disease, instigates serious problems on golf courses by disrupting the uniformity and aesthetic value of putting greens (4). The disease is most severe in areas with closely mowed grasses (35) and low nitrogen (25). *S. homoeocarpa* occurs in the fall through the spring, but is especially active during periods with warm daytime temperatures, high humidity, and cool nights. These conditions provide a humid leaf canopy which, in addition to dry root systems, creates a conducive environment for development of dollar spot (7). Dormant mycelium or stromata that survive in turfgrass thatch or soil resume growth toward the foliage canopy at temperatures of 10 to 32.2°C (5) whereas, at temperatures above 32.2°C, fungal growth can be suppressed (29).

The host range of *S. homoeocarpa* includes species of both warm- and cool-season turfgrasses (25). Leaf symptoms of dollar spot first appear as yellow spots and expand outward to form hourglass-shaped lesions on infected grass blades (29). Lesions turn light-brown with dark-brown borders and, eventually, the entire leaf becomes bleached (33). A small, sunken, brown patch forms in the turf canopy as the infection spreads to other leaves (29).

Control of dollar spot is a high priority for golf course managers, and several measures can be utilized to reduce damage from the disease. Dollar spot may be prevented by avoiding conditions of extremely low nitrogen (7), using resistant cultivars (3), maintaining proper irrigation and mowing practices (1,21,30), and spreading guttation water accumulations by light-weight rolling (23). Throughout the world, more money is spent for chemicals to control dollar spot than any other turfgrass disease (33). Overuse of fungicides has lead to survival of fungicide-resistant *S. homoeocarpa* isolates (14). As a result, dollar spot has become a persistent problem for golf course managers.

Vegetative compatibility, the ability of two isolates to form a heterokaryon through fusion of hyphae, has been tested among isolates of *S. homoeocarpa* from Michigan, Illinois, and Wisconsin. Of these isolates, which were believed to be involved with seasonal infection of dollar spot, six vegetative compatibility groups (VCGs) were described (26). In addition, the same VCG strains (26) were used by Viji et al. (34) to delineate isolates from various areas of the United States and Canada. In this study (34), these isolates were successfully separated into their known VCGs by amplified fragment length polymorphism (AFLP). Previous studies have indicated a correlation between pathogenicity and VCGs (18,19,34). Viji et al. (34) also performed pathogenicity assays and correlated VCGs with virulence of *S. homoeocarpa* isolates on creeping bentgrass.

Isolates of *S. homoeocarpa* from golf courses in Tennessee and northern Mississippi had resistance to either iprodione, thiophanate-methyl (benzimidazole precursor), or propiconazole individually as well as resistance to multiple fungicides (2). The objectives for the current study were as follows: (i) determine whether or not fungicide resistance of these isolates from Tennessee and Mississippi can be associated with specific VCGs; (ii) evaluate genetic diversity among isolates at the nucleotide level by sequencing regions of the conserved genes for elongation factor 1-α (EF1-α), β-tubulin, carbomoylphospate synthase (CPS) domain of the CAD complex, aspartate transcarbamylase (ATC), and dihydroorotase (DHO) and internal transcribed spacer (ITS) regions 1 and 2 (ITS1 and -2); (iii) correlate molecular and VCG differences with fungicide resistance; and (iv) determine whether cluster analysis of AFLP molecular markers could be associated with VCG tester group members or fungicide resistance.

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MATERIALS AND METHODS

Vegetative compatibility. Depending on resistance or susceptibility to various fungicides, five isolates from each of the 10 collection sites from Tennessee and Mississippi (2) as well as four isolates with known fungicide resistance received from Michigan State University (Table 1) were chosen for vegetative compatibility studies. Inoculum for each isolate was grown in the dark at 22°C on Difco potato dextrose agar (PDA; Becton, Dickenson, and Co., Sparks, MD) in 60-by-15-mm petri dishes (Fisherbrand, Atlanta, GA) for 1 week. Mycelial plugs were cut with sterile plastic soda straws and placed approximately 1.5 cm apart on PDA dishes. All pairings were incubated at 26°C for 1 week with 12-h light and dark periods (17) in a Low Temperature Illuminated Incubator 818 (Precision Scientific, Chicago). The presence of a dark barrage zone between the pairings was scored as incompatible and confluent hyphal growth scored as compatible (26). The interaction of the isolates also was examined microscopically. Incompatible reactions between pairs were not repeated. Compatible isolates were cultured twice for confirmation. All isolates were paired with themselves and with all other isolates in addition to the six VCG tester isolates (26).

Table 1. Isolates of *Sclerotinia homoeocarpa* used in this study and their origin, fungicide resistance, and original host cultivar

Isolate	Golf course	Location	Fungicide resistance[a]	Bentgrass cultivar
BM4	Bays Mountain	Seymour, TN	N	Penncross, L 93, Crenshaw
BM7	Bays Mountain	Seymour, TN	N	Penncross, L 93, Crenshaw
BM8	Bays Mountain	Seymour, TN	N	Penncross, L 93, Crenshaw
BM9	Bays Mountain	Seymour, TN	N	Penncross, L 93, Crenshaw
BM10	Bays Mountain	Seymour, TN	I	Penncross, L 93, Crenshaw
CH1	Cherokee	Olive Branch, MS	T	Crenshaw
CH2	Cherokee	Olive Branch, MS	T	Crenshaw
CH3	Cherokee	Olive Branch, MS	T	Crenshaw
CH4	Cherokee	Olive Branch, MS	T	Crenshaw
CH5	Cherokee	Olive Branch, MS	T	Crenshaw
T1	Cottonwoods	Robinsville, MS	I	Crenshaw
T2	Cottonwoods	Robinsville, MS	I	Crenshaw
T3	Cottonwoods	Robinsville, MS	I, T	Crenshaw
T6	Cottonwoods	Robinsville, MS	I, T	Crenshaw
T9	Cottonwoods	Robinsville, MS	I	Crenshaw
G2	Gettysvue	Knoxville, TN	T	Crenshaw
G3	Gettysvue	Knoxville, TN	T	Crenshaw
G4	Gettysvue	Knoxville, TN	T	Crenshaw
G5	Gettysvue	Knoxville, TN	T	Crenshaw
G8	Gettysvue	Knoxville, TN	T	Crenshaw
L1	Legends	Franklin, TN	I	Pennlinks
L2	Legends	Franklin, TN	I	Pennlinks
L3	Legends	Franklin, TN	N	Pennlinks
L4	Legends	Franklin, TN	I	Pennlinks
L5	Legends	Franklin, TN	N	Pennlinks
L1-1	Little Course 1	Franklin, TN	I	18th Green
L1-2	Little Course 1	Franklin, TN	I	18th Green
L1-3	Little Course 1	Franklin, TN	I	18th Green
L1-4	Little Course 1	Franklin, TN	I	18th Green
L1-5	Little Course 1	Franklin, TN	I	18th Green
L2-1	Little Course 2	Franklin, TN	N	SR 1020
L2-2	Little Course 2	Franklin, TN	I	SR 1020
L2-3	Little Course 2	Franklin, TN	N	SR 1020
L2-4	Little Course 2	Franklin, TN	I	SR 1020
L2-5	Little Course 2	Franklin, TN	I	SR 1020
W5	Willow Springs	Athens, TN	T, P, I	Crenshaw
W6	Willow Springs	Athens, TN	T, P, I	Crenshaw
W7	Willow Springs	Athens, TN	T, P, I	Crenshaw
W8	Willow Springs	Athens, TN	T, P, I	Crenshaw
W10	Willow Springs	Athens, TN	T, P, I	Crenshaw
WW1	Whispering Woods	Olive Branch, MS	T	Crenshaw and L93
WW2	Whispering Woods	Olive Branch, MS	T	Crenshaw and L93
WW3	Whispering Woods	Olive Branch, MS	T	Crenshaw and L93
WW4	Whispering Woods	Olive Branch, MS	T	Crenshaw and L93
WW5	Whispering Woods	Olive Branch, MS	T	Crenshaw and L93
M1	Memphis National	Memphis, TN	N	Crenshaw
M2	Memphis National	Memphis, TN	N	Crenshaw
M3	Memphis National	Memphis, TN	N	Crenshaw
M4	Memphis National	Memphis, TN	N	Crenshaw
M5	Memphis National	Memphis, TN	N	Crenshaw
MID	Unknown	Michigan	I	Unknown
MIB	Unknown	Michigan	T	Unknown
MIDMI	Unknown	Michigan	P	Unknown
MIW	Unknown	Michigan	N	Unknown
VCG A	Unknown	Michigan	U	Unknown
VCG B	Unknown	Michigan	U	Unknown
VCG C	Unknown	Michigan	U	Unknown
VCG D	Unknown	Michigan	U	Unknown
VCG E	Unknown	Michigan	U	Unknown
VCG F	Unknown	Michigan	U	Unknown

[a] Fungicide resistance to the following compounds: N = None, I = Iprodione, T = Thiophanate-methyl, P = Propiconazole, and U = Unknown.

Conserved gene amplification. Isolates were cultured in 30 ml of sterile Difco Bacto nutrient broth (Difco Laboratories, Detroit) contained in stationary 125-ml Erlenmeyer flasks for 7 days at 22°C. Mycelia were separated from the medium by centrifugation (approximately 4,000 × *g*) and then frozen at –80°C. Samples were ground using an autoclaved mortar and pestle with liquid nitrogen. DNA was extracted using Qiagen DNeasy Plant DNA isolation kit (Qiagen, Valencia, CA) protocol and eluted in 10 mM Tris, pH 8.0. Genomic DNA was visually inspected for quality and quantified by agarose gel electrophoresis using Low Mass DNA Ladder (Invitrogen, Carlsbad, CA). All polymerase chain reactions (PCRs) contained the following components: 2 µl of dNTPs (2 mM; Eppendorf, Westbury, NY), 2 µl of PCR buffer (10×), 2 µl of forward primer (30 µM), 2 µl of reverse primer (30 µM; Integrated DNA Technologies, Coralville, IA), 1.2 µl of $MgCl_2$ (25 mM), 0.8 µl of AmpliTaq Gold with GeneAmp (5 U/µl), 7 µl of water, and 2 ng of genomic DNA. Buffer and $MgCl_2$ were provided with the AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR products were separated electrophoretically on 1% agarose gels prepared with 1× Tris-acetate-EDTA buffer amended with ethidium bromide and run at 100V for 60 min. A 100- to 2,686-bp eXACTGene Cloning DNA Ladder (Fisher, Atlanta) or a 50- to 1,000-bp BioMarker Low DNA Ladder (BioVentures, Murfreesboro, TN) was used as a size standard for PCR products. A portion of Ef1-α was PCR amplified using degenerate primers 526F (5′GTC GTY ATY GGH CAY GT3′) and 1567R (5′AC HGT RCC RAT ACC ACC SAT CTT3′) (27). A modified touchdown PCR program was as follows: initial denaturation of 95°C for 8 min; 10 cycles of 95°C for 15 s, 66°C for 20 s decreasing by 1°C each cycle, and 72°C for 1 min 30 s; 32 cycles of 95°C for 15 s, 56°C for 2 min, and 72°C for 1 min 30 s; and a final extension of 72°C for 3 min and 4°C hold (33). Glass and Donaldson (8) primer sequences for Bt2a (5′GGT AAC CAA ATC GGT GCT GCT TTC3′) and Bt2b (5′ACC CTC AGT GTA GTG ACC CTT GGC3′) were used to amplify β-tubulin. A modified thermal cycler program consisted of an initial denaturation of 94°C for 8 min; 32 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 5 min and 4°C hold (22). Primer sequences that amplify approximately one-half of the CPS region of CAD, 54F/405R, and 338F/680R were obtained from Moulton and Wiegmann (22). Degenerate primer sequences were as follows: 54F (5′GTN GTN TTY CAR CAN GGN ATG GT3′), 405R (5′GCN GTR TGY TCN GGR TGR AAY TG3′), 338F (5′ATG AAR TAY GGY AAT CGT GGH CAY AA3′), and 680R (5′AAN GCR TCN CGN ACM ACY TCR TAY TC3′). Modified thermal cycler program for the primer pair 54F/405R consisted of initial denaturation of 94°C for 5 min; 5 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30 s; followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s; and a final extension of 72°C for 5 min and 4°C hold. A modified thermal cycler program for primer pair 338F/680R consisted of initial denaturation of 94°C for 5 min; 5 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min 30 s; followed by 35 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 1 min 30 s; and a final extension of 72°C for 5 min and 4°C hold (22). Primer sequences to amplify were acquired from White et al. (36) and were as follows: ITS1 (5′TCC GTA GGT GAA CCT GCG G3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC3′). The altered thermal cycler program was as follows: initial denaturation of 95°C for 9 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min and 4°C hold.

All PCR reactions were purified using Qiaquick PCR Purification Kit no. 28106 (Qiagen). For both primer combinations for the CPS portion of CAD, some non-specific annealing of the primers occurred, causing fragments other than the target sequences to be produced. After agarose gel electrophoresis, the target band (approximately 1,000 to 1,200 bp) was excised using a no. 11 scalpel during visualization with UV light. The target fragment then was processed with a Qiaquick Gel Extraction Kit no. 28706 (Qiagen) and eluted in nanopure water.

DNA sequencing. Standard master mixes for each gene were made that contained 3 µl of ABI buffer (5×), 1 µl of forward or reverse primer (3 pM/µl), 1.5 µl of Big Dye, and 5 to 10 ng of template DNA in 20 µl (total volume). All reagents except the primer were supplied with the Big Dye kit (Applied Biosystems). Products of the sequencing reactions were purified using sephadex gel matrix columns injected into the wells of the MJ BaseStation DNA Sequencer (BioRad, Waltham, MA) and electrophoresed using the following conditions: 200 V of pre-run voltage for 1 min, 5,000 V of injection voltage for 60 s, and 2,200 V of run voltage for 20,000 scans. The accompanying Cartographer sequence analysis software was used to track and score lanes, obtain Phred values, and export the sequences in a format compatible with Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI) sequence editing software. Forward and reverse sequences were aligned and nucleotide–nucleotide BLAST searches were performed on the National Center for Biotechnology Information website to confirm the identity of sequences to known genes sequences. Introns were annotated and edited sequences were submitted to GenBank.

AFLP. The procedure described by Vos et al. (35) and modified by Habera et al. (9) was used for AFLP analyses. DNA was extracted as previously described from 60 isolates of *S. homoeocarpa*. Selective reaction primer sets tested included the following: Eco+AC/Mse+CA, Eco+AC/Mse+C, Eco+AT/Mse+C, Eco+TT/Mse+C, Eco+TA/Mse+C, Eco+TG/Mse+C, and (Eco+AA/Mse+C and Eco+TG/Mse+C). AFLP fragments were detected and analyzed on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Electropherograms produced by the CEQ 8000 were analyzed by comparing fragments to a 600-bp CEQ DNA Size Standard using quartic model. The Y threshold for signal strength was set arbitrarily at 5,000 RFU and bin at 1.5 nucleotides. Binary data ("1" representing presence of a fragment and "0" representing absence of a fragment) was edited manually to ensure that the software correctly scored the peaks. Similarity indices were calculated by NTSYSpc (version 2.20b; Exeter Software, E. Setauket, NY) using Jaccard association coefficient. In addition, pairwise absolute distances were determined using phylogenetic analysis using parsimony (PAUP; version 4.0b 10; Sinauer Associates, Sunderland, MA). From the similarity indices, cluster analysis was performed using the unweighted pair group with arithmetic means analysis (UPGMA) method, which produced a tree dendrogram. Bootstrap values were calculated for cluster support using WinBoot (University of Washington and Joseph Felsenstein). Values below 50 were not labeled on the tree, as in Trigiano et al. (31).

RESULTS

Compatibility. All isolates were self-compatible, and isolates were compatible with most other isolates from the same location (Table 2). For example, isolates G1, G2, G3, G4, and G5 all were exclusively compatible within location, as were WW1, WW2, WW3, WW4, and WW5. However, examples of exceptions include CH4 that was only compatible with CH2 and CH3, but not CH1 or CH5, although all isolates were collected from Olive Branch, MS. Other exceptions included isolate T1, which was not compatible with any of the isolates from location T, whereas T2, T3, T6, and T9 were compatible with each other. The isolate L1 was only compatible with L4 and isolates L1-1 and L1-4 were only compatible with each other and L1-5, suggesting that L1 and L4 are genetically different than the majority of isolates from Franklin, TN.

Isolates, such as BM4, BM7, BM8, T1, L1-2, L1-3, M1, MIB, MIDMI, and MIW, were incompatible with all isolates except themselves. Some isolates were compatible with isolates from different locations. For example, W isolates were compatible with several isolates from L, L1, and L2. W isolates were collected in Athens, TN, whereas L, L1, and L2 were from different courses in Franklin, TN.

Sixteen Tennessee and Mississippi isolates were compatible with tester strains. Isolates from L, L1, L2, and W were compatible with tester strains A, B, and D. Two isolates (G4 and G8) were compatible with VCG tester strain C and only one isolate (MIB) was compatible with VCG tester strain F. Most isolates that were compatible with each other also were in a common VCG, with a few exceptions. For example, isolate T6 was not compatible with any tester strains but was compatible with sample L2-1 that was classified as being in VCGs A, B, and D. Another example is Michigan isolates MIW and MIB, which were in VCG D, but not compatible with any other isolate in that group. Isolates G4 and G8 both were found to be in VCG C and compatible with each other, yet no other isolate from that location was compatible with VCG C. Tester strains A, B, and D were compatible with each other.

Conserved gene sequence analysis. Sequences for all of the isolates were identical for all four gene regions. EF1-α PCR products were approximately 1,100 bp, whereas the CAD 338F/680R and 54F/405R PCR products were approximately 1,000 and 1,200 bp, respectfully. β-tubulin primers produced a 500-bp PCR product and ITS primers produced a 550-bp PCR product. After sequencing, the slight overlap of CAD 338F/680R and 54F/405R PCR products were aligned to form a contiguous 1,820-bp sequence.

Intron and exon annotations and GenBank Accession IDs are described in Table 3. One intron was detected in the β-tubulin fragment as well as in the CAD fragment. Estimated location of introns and primer annealing sites for each gene are illustrated in Figure 1. During nucleotide–nucleotide BLAST searches, all genes matched the appropriate sequences except the CAD fragment. The CAD sequence was confirmed by alignment with the known CAD sequence of a *Discula* sp. (Dr. John K. Moulton, University of Tennessee, *personal communication*).

Association of AFLP markers, vegetative compatibility, and fungicide resis-

tance. Eco+TG/Mse+C and Eco+AA/Mse+C primer combinations were the most informative by yielding fragments that ranged between 51 and 711 and 55 and 620 bp, respectively, during capillary gel electrophoresis. Fragments above 600 bp were excluded from the study due to low fluorescence. The Eco+TG/Mse+C primer combination produced 44 polymorphic fragments, whereas Eco+AA/Mse+C produced 62 polymorphic fragments.

Similarity indices were calculated from the binary data, which ranged between 0.86 and 1.00. The most diverse (0.86) isolates were L1-2/L2-2. Similarity indices within locations ranged as follows: BM, 0.94 to 1.00; CH, 0.94 to 1.00; T, 0.99 to 1.00; G, 0.95 to 0.99; L, L1, and L2, 0.86 to 1.00; W, 0.96 to 1.00; WW, 0.96 to 1.00; M, 0.97 to 1.00; MID, MIB, MIDMI, and MIW, 0.92 to 0.97; and VCG A, B, C, D, E, and F, 0.91 to 0.99. Pairwise absolute character differences ranged from 0 to 14 fragments, indicating some isolates that were the same at all AFLP loci.

Cluster analysis of similarity indices produced two major groups (Figs. 2 and 3, groups 1 and 2). Four smaller subgroups also were formed and labeled with letters

Table 2. Vegetative compatibility pairings of *Sclerotinia homoeocarpa* isolates and their vegetative compatibility group (VCG) and fungicide resistance

Isolate	Compatible isolates	VCG[a]	Fungicide resistance[b]
BM4	BM4	–	N
BM7	BM7	–	N
BM8	BM8	–	N
BM9	BM9, BM10	–	N
BM10	BM9, BM10	–	I
CH1	CH1, CH2, CH3, CH5	–	T
CH2	CH1, CH2, CH4, CH5	–	T
CH3	CH1, CH3, CH4, CH5	–	T
CH4	CH2, CH3	–	T
CH5	CH1, CH2, CH3, CH5	–	T
T1	T1	–	I
T2	T2, T3 T6, T9, L3, W6, W7	–	I
T3	T2, T3, T6, T9	–	I, T
T6	T2, T3, T6, T9, L2-1, W6, W7, W10	–	I, T
T9	T2, T3, T6, T9	D	I
G2	G2, G3, G4, G5, G8	–	T
G3	G2, G3, G4, G5, G8	–	T
G4	G2, G3, G4, G5, G8	C	T
G5	G2, G3, G4, G5, G8	–	T
G8	G2, G3, G4, G5, G8	C	T
L1	L1, L4	–	I
L2	L2, L3, L4, L5, L21, L2-2, L2-4, L2-5, W5, W6, W7	A,B,D	I
L3	T2, T6, L2, L3, L4, L5, W5, W6,W7. MID	A,B,D	N
L4	L1, L2, L3, L4, L5, L2-4, W5, W6, W7, MID	A,B,D	I
L5	L2, L3, L4, L5, L2-1, L2-2, L2-4, L2-5	B,D	N
L1-1	L1-1, L1-4, L1-5	–	I
L1-2	L1-2	–	I
L1-3	L1-3	–	I
L1-4	L1-1, L1-4, L1-5	–	I
L1-5	L1-1, L1-4, L1-5	–	I
L2-1	T6, L2, L3, L4, L5, L2-1, L2-2, L2-3, L2-4, L2-5, W5,W6, W7	A,B,D	N
L2-2	L2, L3, L4, L5, L2-1, L2-2, L2-3, L2-4, L2-5, W5,W6, W7	A,B,D	I
L2-3	L2-1, L2-2, L2-3, L2-5	–	N
L2-4	L2, L3, L4, L2-4, W8, W10	A,B,D	I
L2-5	L2, L3, L4, L2-1, L2-2, L2-3, L2-4, L2-5, W5,W6, W7	A,B,D	I
W5	L2, L3, L4, L2-1, L2-2, L2-5, W5 W6, W7, W8	A,B,D	T, P, I
W6	T2, T6, L2, L3, L4, L2-1, L2-2, L2-5, W5 W6, W7, W8, W10	A,B,D	T, P, I
W7	T2, T6, L2, L3, L4, L2-1, L2-2, L2-5, W5 W6, W7, W8, W10	A,B,D	T, P, I
W8	L2-4, W5, W6, W7, W8, W10	–	T, P, I
W10	L2-4, T6, W6, W7,W8, W10	–	T, P, I
WW1	WW1, WW2, WW3, WW4, WW5	–	T
WW2	WW1, WW2, WW3, WW4, WW5	–	T
WW3	WW1, WW2, WW3, WW4, WW5	–	T
WW4	WW1, WW2, WW3, WW4, WW5	–	T
WW5	WW1, WW2, WW3, WW4, WW5	–	T
M1	M1	–	N
M2	M2, M4	–	N
M3	M3, M5	–	N
M4	M2,M4, M5	–	N
M5	M3, M4, M5	–	N
MID	L3, L4, MID	–	I
MIB	MIB	D,F	T
MIDMI	MIDMI	–	T, P
MIW	MIW	D	N
VCG A	L2, L3, L4, L2-1, L2-2, L2-4, L2-5, W5, W6, W7	A, B, D	U
VCG B	L2, L3, L4, L5, L2-1, L2-2, L2-4, L2-5, W5, W6, W7	A, B,D	U
VCG C	G4, G8	C	U
VCG D	T9, L2, L3, L4, L5, L2-1, L2-2, L2-5, L2-4, W5, W6, W7, MID	A,B,D	U
VCG E	None	E	U
VCG F	MIB	F	U

[a] VCGs A–F (26); – indicates incompatible with all tester strains tested.
[b] Fungicide resistance to the following compounds: N = None, I = Iprodione, T = Thiophanate-methyl, P = Propiconazole, and U = Unknown.

A, B, C, and D. Fungicide resistance (Fig. 2) and VCG (Fig. 3) were overlaid on the AFLP tree. Some isolates from the same location clustered together; WW isolates in subgroup A and L, L1, and L2 isolates in subgroup C. Some isolates from the same location did not group together. For instance, isolates BM4 and BM10 were in group 1 whereas BM9 was in group 2. Most bootstrap values calculated by WinBoot were less than 50, which indicated little support for almost all clusters in the dendrogram, and were omitted, with a few exceptions. Sufficient bootstrap values ranged between 52 and 99.

DISUSSION

Assessment of vegetative compatibility is a classical genetic technique for identifying related subpopulations within a species. Isolates from within a location typically were compatible with each other, which supports the hypothesis that they are from the same source and therefore genetically similar. Vegetative compatibility pairings and characterization into VCGs also has been completed using *Verticillium dahliae* Kleb. isolates from around the world (18). Genetic homogeneity within a location and not among locations was described and appears to be similar to the diversity revealed among *S. homoeocarpa* isolates. Low diversity of both *V. dahliae* and *S. homoeocarpa* can be explained by the lack of sexual reproduction. For *S. homoeocarpa*, low genetic diversity also is enhanced by the absence of asexual reproduction by spores. Dispersal of different genotypes via mycelium fragmentation is limited and would not support combination of genetically dissimilar isolates. The slight diversity among isolates as indicated

Table 3. Length, exon and intron positions, and GenBank accession numbers for *Sclerotinia homoeocarpa* gene sequences[a]

Gene	Length (bp)	Exon positions (bp)	Intron position (bp)	GenBank accession no.
EF1-α	974	64–122, 317–974	1–63, 123–316	DQ448301
β-tubulin	424	1–106,199–424	107–198	DQ448299
CPS	1,817	1–454, 476–1,817	455–475	DQ448300
ITS1, 5.8S, ITS2	542	N/A	N/A	DQ448302

[a] EF = elongation factor, CPS = carbomoylphospate synthase, ITS = internal transcribed spacer, and N/A = not applicable.

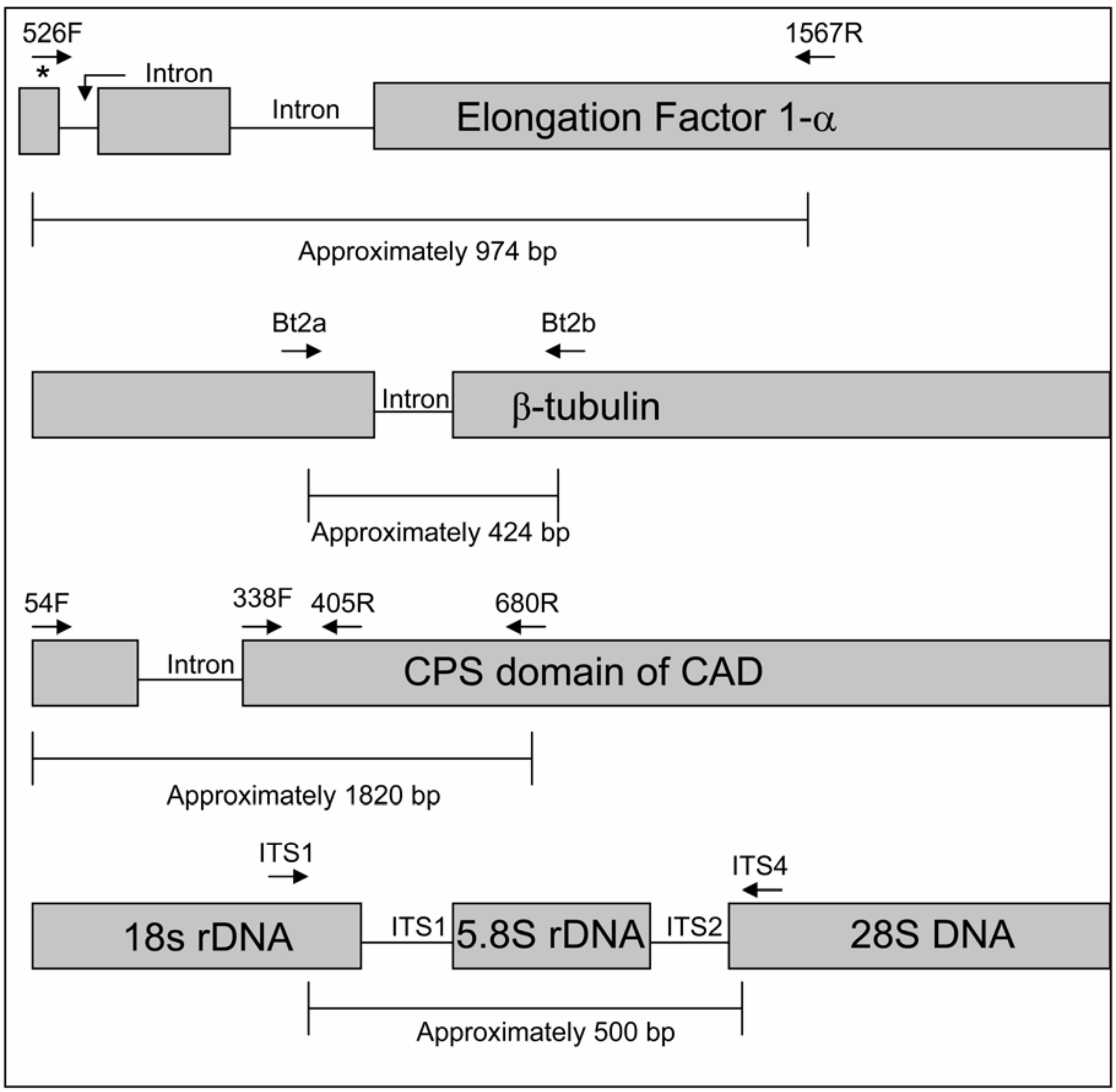

Fig. 1. Estimated intron and primer annealing sites on *Sclerotinia homoeocarpa*-conserved genes. Elongation factor 1-α (modified from Rehner and Buckley [27]), β-tubulin (modified from Glass and Donaldson [8]), carbomoylphospate synthase (CPS) domain of CAD (modified from Moulten and Wiegmann [22]), and internal transcribed spacer (ITS; modified from White et al. [36]); an asterisk (*) indicates area where an exon matched the degenerate primer site. These sequences, along with other unresolvable nucleotides, were removed during sequence editing and not included in the final base pair count.

by AFLP analyses could be ascribed to random mutations or, equally likely, the resolution limitations of the AFLP technique.

Compatibility of isolates within a specific location could be explained by founder effects. Founder effects or genetic bottlenecks occur when a population is initiated by an individual or a limited number of individuals that do not represent the genetic diversity found in the parent population. It is possible that one isolate of *S. homoeocarpa* was separated from a larger original population and all isolates within the new location could have been derived from the original progenitor. Multiple isolates with compatibility at the same location could be considered clones from an original isolate that spread due to cultural practices and foot traffic (10). Purchasing contaminated turfgrass seed or sod from the same source could explain this genetic similarity at the vegetative compatibility level among Tennessee and Mississippi locations. Because the inoculum of *S. homoeocarpa* is passively dispersed by contaminated equipment, the disease is easily spread within golf courses (6).

Vegetative compatibility of *S. homoeocarpa* isolates also has been tested among isolates from Michigan, Illinois, and Wisconsin. Of these isolates, which were believed to be involved with seasonal infection of dollar spot, six VCGs were delineated (26). Most of the samples collected from these sites over the 3-year study were primarily in VCGs A and B. Most isolates from L, L1, L2, and W were compatible with tester strains A, B, and D. In contrast, most isolates included in other studies were found to be in VCG A or VCG B, but not VCG D (6,26,34). Selection pressures, such as fungicide usage, may support the dominance of one genotype over all others (11). However, a significant correlation could not be made among fungicide resistance location and VCG in the current study. Some isolates in groups A, B, and D lacked resistance and others were resistant to multiple fungicides. Therefore, these results do not support the hypothesis that fungicide resistance is associated with VCGs.

Most studies of vegetative compatibility with *S. homoeocarpa* did not indicate that isolates were compatible with multiple tester strains (6,26,34). The same VCG tester isolates that were used by Viji et al. (34) were used in our studies and, as expected, tester strains A, B, and D were not compatible with each other. In this study, most isolates were compatible with multiple tester strains, especially A, B, and D. Isolates being compatible with more than one tester strain also were reported from pairing of isolates from the eastern United States and Canada (34). In this study, a single Canadian isolate was compatible with VCG C and D. Isolates classified into more than one VCG implies that these tester strains are not as specific as first thought, or that the tester strains changed genetically during storage or possibly are mislabeled.

Sequencing of the β-tubulin gene yielded a fragment of approximately 424 bp with one intron. In contrast, Glass and Donaldson (8) found three introns in the 495-bp fragment using the same primers as with *Neurospora crassa*. The portion of EF1-α amplified by the same primers used in this study was found to be about the same length (1,200 bp) as *Beauveria* isolates (28). Four introns were detected in this portion of EF1-α in *Beauveria* spp., but only two were found in the current study. An exon exists before the first intron, but it was removed because it was a degenerate primer annealing site. About one-half of the CPS domain of CAD was isolated in this experiment and contained one intron. Originally, the primers for amplification of CAD were designed for insect studies (22), and their successful use in this study implies high conservation of the regions where primers anneal. The combined length of ITS1, 5.8S rDNA, and ITS2 were comparable with those provided by White et al. (36).

Lack of variation in conserved gene sequences and ITS regions strongly indicates that very little diversity exists between isolates and agrees with Powell and Vargas (26) that comparison of ITS1 regions of *S. homoeocarpa* isolates indicated no diversity among isolates from different locations. ITS sequences are not under selec-

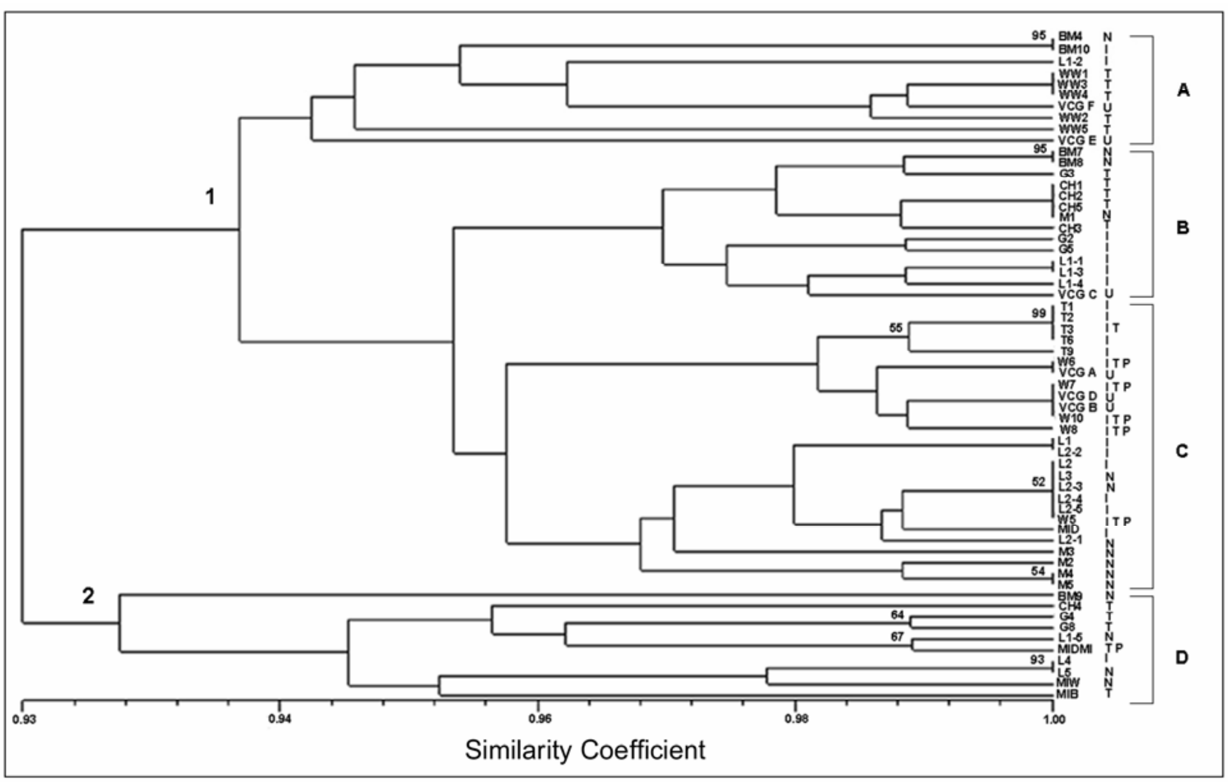

Fig. 2. Dendrogram produced from calculated similarity indices of *Sclerotinia homoeocarpa* isolates. Some isolates cluster according to fungicide resistance and by location. Fungicide resistance is indicated to the right of the isolate name. Key for fungicide abbreviations: N = none, I = iprodione, T = thiophanate-methyl, U = unknown, and P = propiconazole. Numbers at nodes equal bootstrap values (only values greater than 50 are shown).

tion pressure because they do not code for proteins; thus, a large amount of diversity would be expected in this region when studying various organisms. All other genes sequenced in this study are essential to cell function, and we expected to detect some variation among isolates. β-tubulin has been known as a target for benzimidazole (thiophanate-methyl is a precursor for benzimidazole) in roundworms and fungi. Studies have examined associations between benzimidazole activity and β-tubulin sequence. Some residues of the β-tubulin sequence were correlated to benzimidazole vulnerability. Those particular residues were proposed to be involved in benzimidazole binding (15). Comparison of field-mutated and laboratory-mutated strains of *Venturia inaequalis* indicated that changing of important codons in β-tubulin gene sequences provided benomyl resistance to the isolate (16). Lack of variation at all loci supports the hypothesis that *S. homoeocarpa* populations in Tennessee and northern Mississippi, and perhaps populations from other regions, are from an original founding population, which subsequently has been dispersed through dissemination of vegetative mycelium.

AFLP has proven to be a useful technique for assessing genetic diversity in *S. homoeocarpa*. Cluster analysis (UPGMA) of AFLP loci indicated that isolates did not group according to fungicide resistance. In subgroups A, B, C, and D, an array of fungicide resistance was illustrated. For instance, in subgroup C, many isolates demonstrated iprodione resistance in addition to thiophanate-methyl and propiconazole resistance, and some had no resistance to any fungicide. Bootstrap values were insufficient to statistically support subgroup branches and therefore, there is little confidence in the apparent clustering of isolates. Clusters with sufficient bootstrap support were not consistently resistant to a single fungicide. For instance, the branch with BM4/BM10 and L4/L5 had bootstrap values above 90, which indicated strong support to the grouping, but there were two different types of fungicide resistance. A similar study (12) using UPGMA and principle component analysis to relate random amplified polymorphic DNAs (RAPDs) to host specificity also was not able to clearly expose a relationship between genotype and resistant phenotype. Some clustering occurred for isolates with similar host specificity, but the significance was difficult to interpret.

In contrast to fungicide resistance, isolates within a similar VCG appeared to cluster together in subgroups. For example, in subgroup C, isolates that were compatible with VCG A, B, and D clustered together as well as tester strains A, B, and D. Another study (34) has shown similar results in the respect that isolates in the same VCG grouped on a dendrogram. The same VCG tester strains were used by Viji et al. (34) and isolates were separated successfully into their known VCGs. In the current study, isolates G4 and G8 grouped together in subgroup D, but did not cluster with tester strain C in subgroup B. Viji et al. (34) also found that isolates in VCG C separated into two different subgroups. Only one primer combination (Eco+AG/Mse+C) was used (34), whereas two different pairs were used in this study. During preliminary primer trials in this study, no polymorphisms were identified using this Eco+AG primer set. Regions of DNA associated with the genes for compatibility may be associated with these primer combinations.

In addition to isolates clustering into VCGs on the dendrogram, isolates appeared to be grouping by location, although bootstrap values were low. Some isolates, such as BM9, did not group with other members from the same location. Most of the isolates from location L, L1, and L2 clustered together in subgroup C as well as isolates from WW, T, CH, and M. RFLP of the intergenic spacer region and RAPD analysis also have indicated that isolates from Ontario, Canada, clustered according to population origin with a few exceptions (13). In addition, the similarity calculations among Tennessee and Mississippi isolates ranged between 0.86 and 1.00, which also is similar to the results found among Ontario isolates by Hsiang and Mahuku (13) and Viji et al. (34). This lack of variability is thought to be due to the lack sexual recombination, which again

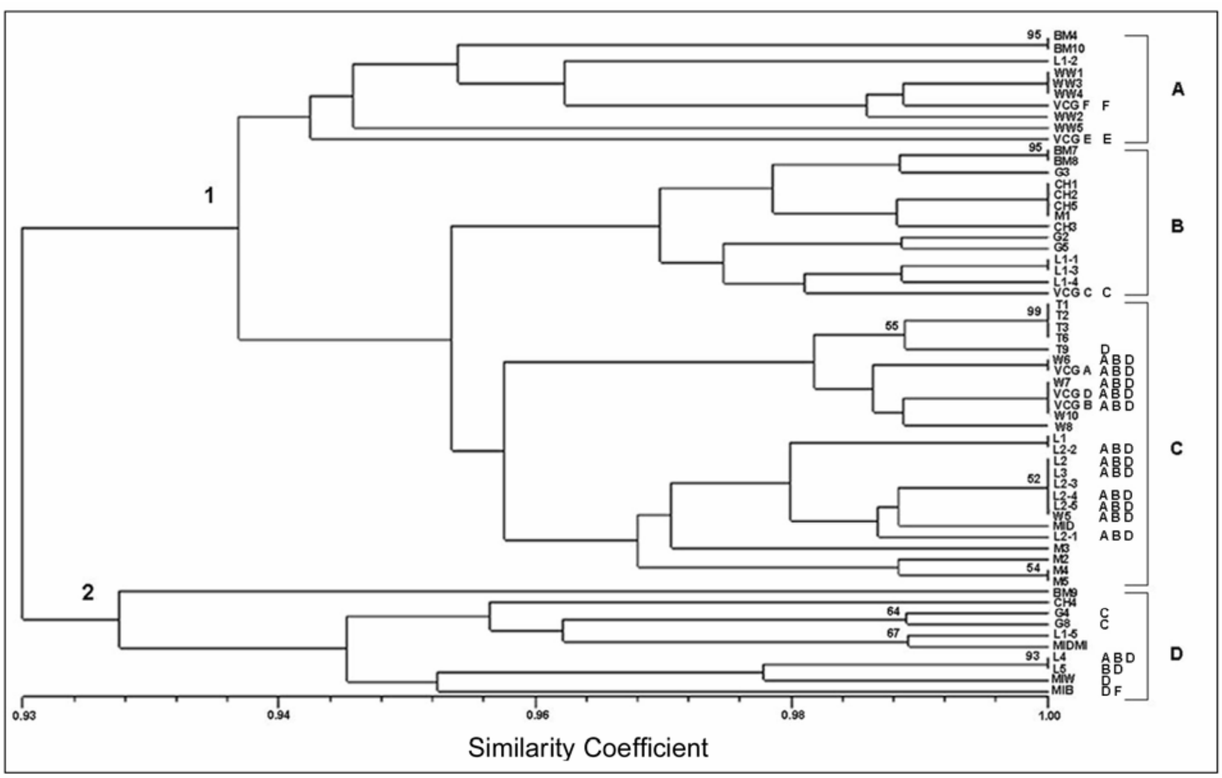

Fig. 3. Dendrogram produced from calculated similarity indices of *Sclerotinia homoeocarpa* isolates. Isolates in vegetative compatibility groups (VCGs) A, B, D, and C group together, with a few exceptions. Vegetative compatibility tester strains (A–F) with which the isolate was compatible are indicated to the right of the isolate name.

indicates that these populations were clonal in nature (13,34). Grouping of isolates from one location during cluster analysis also could be explained by the founder effect, where one isolate of *S. homoeocarpa* initiated the population at a location.

Arbitrarily primed PCR profiling, such as AFLP, have been popular techniques that use indiscriminate or semi-indiscriminate primers for amplification of DNA products (24). Although arbitrary molecular markers are considered to be more efficient than sequencing, little specific information is created about the alleles. Theoretically, AFLPs have two alleles per locus, one that was amplified and one that was not, and genetic differences are not known (20). Additionally, arbitrarily primed PCR techniques do not give information about which region of genomic DNA was amplified. For example, members of the same VCG clustered together in the dendrogram, indicating that AFLP primers could be amplifying regions of DNA associated with the genes for vegetative compatibility. Similarity indices indicated low diversity among Tennessee and Mississippi isolates; therefore, it could be assumed that bands that comigrate are the same (32).

Genetic diversity among Tennessee and Northern Mississippi *S. homoeocarpa* isolates was investigated using vegetative compatibility, conserved gene sequences, and AFLP. Isolates generally were compatible with isolates from the same location, and tester strains were compatible with 16 Tennessee and Mississippi isolates. Portions sequenced of CAD, EF1-α, β-tubulin, and ITS revealed 100% homology among isolates. In addition, 86 to 100% similarity was detected among isolates during UPGMA analysis of AFLP fragments. Fungicide resistance could not be associated with a particular VCG or branch on the dendrogram, although some isolates clustered according to VCGs and location. Low variability of these isolates probably is due to the lack of sexual recombination as well as possible founder effects, and support the contention that these populations are clonal in nature.

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